THE USE OF ACRIDINE ORANGE LUMINESCENCE FOR THE STUDY OF THE SECONDARY
STRUCTURE OF NUCLEIC ACIDS

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THE USE OF ACRIDINE ORANGE LUMINESCENCE FOR THE STUDY OF THE SECONDARY STRUCTURE OF NUCLEIC ACIDS

The determination of the degree of spiralization of this or that nucleic acid has an important significance both for establishing the secondary structure of the nucleic acids of various types and for studying those changes of secondary structure which take place in nucleic acids as a result of various external influences on them, for example, the influence of ultra violet (UV) rays, ionizing radiation, temperature or chemical agents. By the degree of spiralization we understand the ratio of the number of nitrogenous bases, making up the complementary pairs of Watson and Crick and responsible for the formation of sectors with a secondary structure of the double spiral type, to the total number of bases.

Up until now the determination of these values was based mainly on the measurements of light absorption at a wave length of 260 mᴀk (hypochromic effect) or the measurements of optical rotation. In the present work the basis is presented for the possibility of using for this purpose a new method, based on the differences in luminescent

1.

characteristics (duration of fluorescence) of the acridine orange

dye (AO), connected with spiralized (two strand) or despiralized

(one strand) sectors of nucleic acid.

In our work $\underline{/1/}$ it was shown that when AO is bound with native

(two strand) DNA the duration of fluorescence of the dye τ has

practically the same values for the entire spectrum of fluorescence.

When measuring the values τ with light filters which give off narrow

spectral bands in the green portion of the spectrum, in the vicinity

of 530 mmk, and in the long wave portion, in the vicinity of 640 mmk,

the corresponding values for this intensity (τ_z and τ_K) correspond

within the limits of accuracy of the measurements. This indicates

that in the given case we are dealing with one electronic band of

radiation, decaying as a whole. In contrast to this, when AO is

bound with denatured DNA the values τ_z and τ_K differ sharply, which

indicates the presence of two different bands in the composition of the

radiation. This is due to different centers of luminescence (figure 1).

With a sufficiently high concentration of AO on the denatured

DNA, the secondary band is clearly exposed in the spectrum of lumines-

cence and leads to a change in the color of fluorescence from yellowish-

green to red. However, the duration of fluorescence is an incomparably

more delicate indicator of the presence of a secondary band, which

makes it possible to detect it even in those cases when the changes in

the spectra of absorption and fluorescence are still practically im-

possible to catch. $\underline{/1/}$

The table presents a comparison of the main luminescent and absorbtion characteristics of monomer molecules of AO in aqueous solutions, of the green and red bands of fluorescence of the same dye, bound with DNA, and dimers cf the dye (based on data from the works [1; 2].) In the work /1/ it was shown that when bound with native DNA the fluorescent molecules of the dye are monomers, while when bound with denatured DNA there takes place a more or less complete dimerization of the bound dye. This explains the existing difference in the dependency of the values of τ_z and τ_K on the concentration of DNA in cases of native and denatured DNA. In the first case the value τ for a specific concentration of DNA is determined only by an equilibrium between AO in the solution and in the adsorbed condition; in the second -- the curve for τ_z reflects an equilibrium between the AO in the solution and that part of the adsorbed dye which preserved the monmeric form, and the curve for τ_K reflects an equilibrium between molecules of three types: Free monomers in solution, monomers bound on DNA, and bound dimers.

Imagine that we have a mixture of native (two strand) and denatured (one strand) DNA, in which the relative content of the latter is D , and the coefficient of dimerization. that is, the ratio of the number of bound molecules found in the dimeric form to the total number of molecules bound on denatured DNA, equals K . Then, as it is not difficult to see, the value τ_K is determined by the expression

$$\tau_K = \tau_D \frac{a\left[KD(1-I_M\tau_M/I_D\tau_D)+I_M\tau_M/I_D\tau_D\right]+(1-a)I_M'\tau_M'/I_D\tau_D}{a\left[KD(1-I_M/I_D)+I_M/I_D\right]+(1-a)I_M'I_D} \quad .$$

(1)

3.

Here I_M, I'_M and I_D -- are the calculated contributions for one molecule in the luminescence observed in the given spectral band correspondingly of bound monomer, free monomer and bound dimer molecules; τ_M, τ'_M and τ_D -- values for the duration of the stimulated conditions for the same conditions of the AO molecules; a -- the "Coefficient of distribution" of the dye, that is, the fraction of molecules found in a bound condition with DNA.

In a particular case, when $I_M / \bar{I}_D = 1$, the value of the relative change $\overline{\Delta \tau_k}$ for the given mixture precisely equals the percentage content of denatured DNA (degree of despiralization):

$$\overline{\Delta \tau_k} = \left(\tau_k^{x} - \tau_k^{NAT} \right) \Big/ \left(\tau_k^{DEN} - \tau_k^{NAT} \right) = D.$$

(2)

Generally then $\overline{\Delta \tau_k}$ is a more complex function from D . For its tabulation we take the values of τ_M and τ'_M from the table, and introducing the designation $\delta = I_M / I_D$, we rewrite the formula (1) in the form

$$\tau_k = \tau_D \; \frac{a \left[KD(1-0.2\delta) + 0.2\delta \right] + (1-a)\, 0.04\delta}{a \left[KD(1-\delta) + \delta \right] + (1-a)\, 0.5} \; .$$

Here the ratio I'_M / I_M was taken equal to 0.5 from the relations of the outputs of luminescence and absorption coefficients of free and bound monomers of AO for $\tau_k = 4 \cdot 10^{-9}$ sec. The value $25 \cdot 10^{-9}$ sec. was conditionally taken for τ_D , since the true value of τ_D may be some-

4.

what higher than the maximum measured value $2 \cdot 10^{-9}$ sec. /17/.

As an example, figure 2 depicts the plots of the function $\Delta \tau_K$ (D), calculated according to the formula (2) for $a = 0.5$ and various combinations of values for the parameters K and δ. It is significant here that by varying the values of these parameters, we may obtain plots with a great deal of steepness either in the area of small values of D, or in areas of values for D which are close to unity.

Experimentally we have the possibility of varying the parameter δ by using different filters. It is apparent that δ will be decreased as we will cut out from the spectrum of fluorescence all the longer waved sectors (close to the maximum of fluorescence of dimers). The parameters a and K can be varied by changing the values of the concentration of DNA and AO in the solution. Figure 3 shows a number of such experimentally obtained plots, according to which it is possible to determine the content of denatured DNA in the mixture (the DNA was obtained from phage T2 and its properties were described in the work /17/).

In the case when the plot has the form of a straight line (curve 1, figure 3), the values of the density of the corresponding solution of unstained DNA in a wave length of 260 mmk were measured parallel and the values of the relative change of density (hypochromic effect) were calculated:

$$\overline{\Delta \varepsilon_{260}} = \frac{\left(\varepsilon_{260}^{x} - \varepsilon_{260}^{NAT} \right)}{\left(\varepsilon_{260}^{DEN} - \varepsilon_{260}^{NAT} \right)} .$$

The scope of this value, depicted by the small circles in figure 3, coincides within the limits of accuracy of measurement with the values of $\overline{\Delta \tau_{\kappa}}$. However, the proposed luminescent method in this case is two or three times more sensitive than the method based on the measurements of hypochromism, since the ratio of the values of $\mathcal{E}_{360}$ for denatured and native DNA equals 1.4--1.3, and the ratio of the values of τ_{κ} for these objects reaches 3--4. In practice, based on the hypochromic effect, it is difficult to detect the presence of less than 10% of denatured DNA, while based on the new method an admixture of denatured DNA can be sufficiently reliably detected in quantities of 3--5%.

If work is done under the conditions when the steepness of the calibration plots in the vicinity of the origin of coordinates is increasing (curves of type 2, figure 3), then the sensitivity of the luminescent method attains values of D of around 1%.

Of course in practice there is not as much interest in the determination of the content of denatured DNA in a mixture as in the detection of despiralization of this or that type of nucleic acid as a result of the influence of various external factors.

It is natural to assume that in this case the value of D is connected with the measurable relative change τ (with the value $\overline{\Delta \tau_{\kappa}}$) just as in the case of a mixture, and that the degree of despiralization of the DNA under study can be determined based on calibration plots, constructed for the mixtures of native and denatured DNA.

The validity of such an assumption was verified in an experiment

on the "fusion curves" of DNA.* A solution of native DNA from T2

phage in a concentration of 50 mg/ml (citrate buffer 0.005 M + 0.05 M

NaCl, pH 6.9) was heated up to a specific temperature and maintained

at this temperature for a period of 10 minutes. Then a sample was

taken and rapidly cooled to 0°, after which its absorption at a wave

length of 260 mmk was measured at room temperature. Then it was

mixed with such an amount of AO solution in the same buffer that the

values of the concentrations of the DNA and dye in all cases were the

same. The duration of fluorescence of these solutions was measured

in the spectral range $\sim$ 640 mmk (filter KS-10).

For this case the calibration plots, constructed for the

mixtures of native and denatured DNA, have the form of a straight line.

As is seen from figure 4 (curve 2), here the values of the relative

change of the values $\Delta \mathcal{E}_{260}$ and $\overline{\Delta \tau_{K}}$ in the process of DNA melting

actually agree, and consequently we can follow the process of fusion

of DNA based on changes in the value of $\overline{\Delta \tau_{K}}$ as well as based on

changes in the value of $\overline{\Delta \mathcal{E}_{260}}$.

Curves 1 and 3 on figure 4 are obtained by the same method, but

with different values of ionic strength of the solution and without the

construction of calibration plots. Curve 4 was obtained during the

renaturing of DNA. Here the DNA, which has been preliminarily heated

up to 100°, is cooled slowly and maintained at a specific temperature

for 15 minutes. Then a sample is taken and cooled rapidly at 0°; dying with AO was conducted at room temperature.

In these tests, which had the aim only of checking the feasibility of using the proposed method for measuring the degree of despiralization of nucleic acids, we did not undertake the mission of reaching the maximum sensitivity and detecting the minimum degree of despiralization of DNA. It may be thought that with the selection of the appropriate conditions the sensitivity of this method, as in the case of mixtures, turns out to be several times more superior than previous methods, which makes it possible to measure a sufficiently reliable value of D on the order of 1%. In practice there is a convenient circumstance that with working concentrations of DNA (10--20 mg/ml) the values of τ_K depend very little on the concentration of nucleic acid. The experimental results presented in the work were obtained under the following conditions, accepted by us as standard: pH 6.9; ionic strength 0.08; concentration of dye $5 \cdot 10^{-6}$ M; concentration of DNA 10--20 mg/ml. Under these conditions a determination of the degree of spiralization of transfer RNA $\overline{/3/}$ was made.

Literature

a. Borisova, O. F., Tumerman, L. A., Biofizika (Biophysics), 9, 537, 1964.

b. Morozov, Yu. V., Thesis for a Doctor's Degree, FIAN (Institute of Physics of the USSR Academy of Sciences), 1962.

c. Borisova, O. F., Kiselev, L. L., Tumerman, L. A., Doklady of the USSR Academy of Sciences, 152, 1001, 1963.

Comparison of the basic optical and luminescent characteristics of the
AO monomers and dimers

Table

Luminescent characteristics of monomers and dimers	Monomers		Dimers	
	in aqueous solution	on native DNA	on denatured DNA	based on data of [2]
λ_{max} of absorption, mmk	494	504	~474	464
λ_{max} of fluorescence, mmk	530	530	640	640
Half width of the band of fluorescence, mmk	71	50	116	116
Duration of fluorescence, (τ) sec.	$2 \cdot 10^{-9}$	$5 \cdot 10^{-9}$	$\geq 21 \cdot 10^{-9}$	$20 \cdot 10^{-9}$
Relative quantum yield (ρ)	1	2.5	-	-
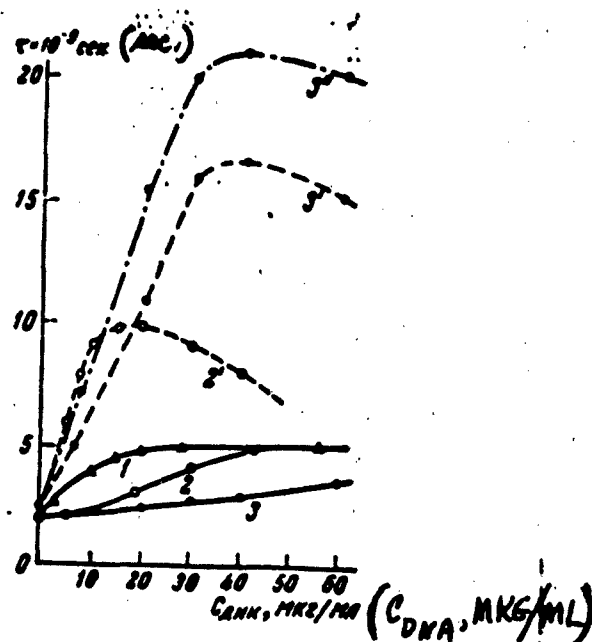
Figure 1. Dependency of τ_k and τ_z for AO on the concentration of native and denatured DNA in the solution.

DNA of phage T2; solvent 0.05 M NaCl + 0.005 M citrate Na; μ = 0.08; pH 6.9; Curve 1 -- native DNA; C_{AO} = 5 $\cdot$ 10^{-6} M. Filter SZS-9 + ZhS-18 (τ_z) or KS-10 (τ_k). Curves 2 and 3 for denatured DNA and concentrations of AO 5 $\cdot$ 10^{-6} M and 2 $\cdot$ 10^{-5} M, filter SZS-9 + ZhS-18 (τ_z). Curves 2' and 3' -- same with filter KS-10 (τ_k). Curve 3" -- denatured DNA; C_{AO} = 2 $\cdot$ 10^{-5} M, filter KS-17 (τ_k).

/Translator's note. The original legend is attached at the left for a comparison of figures. Probable meanings of abbreviations:

z -- green

k -- red

SZS -- blue-green light filter
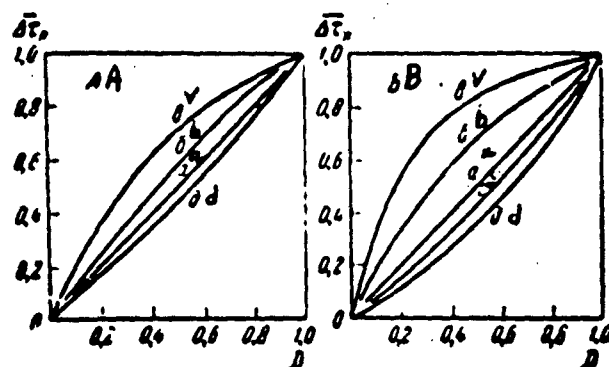
ZhS -- yellow light filter

KS -- red light filter /

Figure 2. Calculated plots of dependency of the value $\overline{\Delta\tau_K}$ on the relative content of denatured DNA in the mixture (D) at $a = 0.5$. On graph A the value of the parameter $K = 0.25$; on graph B -- $K = 0.75$. The curves marked with the letters a, b, v, g, d correspond to the values δ : 1; 0.3; 0.1; 2; 5.

$$\frac{\tau_x \cdot \xi}{\xi \cdot \xi} : \frac{\xi \cdot \xi_N}{\xi \cdot \xi} \qquad \left(\frac{\tau_x - \tau_N}{\tau_D \cdot \tau_N} \; ; \; \frac{\xi_x - \xi_N}{\xi_D \cdot \xi_N} \right)$$

1.00

0.75

0.50

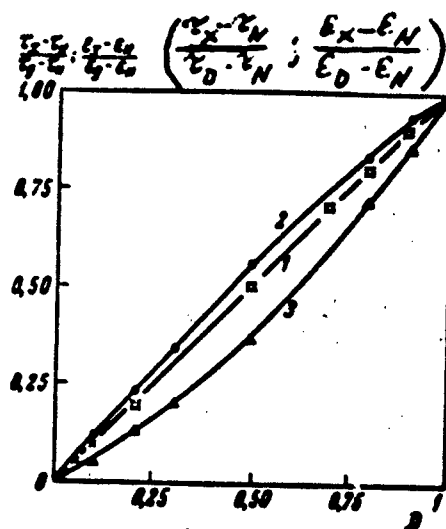*0.25*

0   *0.25*   *0.50*   *0.75* D

Figure 3. Experimental plots of dependency of the relative change for the

value $\Delta \tau_k$ on the content of denatured DNA in the mixture

Curve 1: C_{DNA} = 10 mkg/ml; C_{AO} = 5 • 10^{-6}M; filter KS-10. Curve 2:

C_{DNA} = 5 mkg/ml; C_{AO} = 5 • 10^{-6} M; filter KS-15. Curve 3: C_{DNA} = 5 mkg/ml;

C_{AO} = 5 • 10^{-6} M; filter OS-13. On curve 1 the little circles are the plots

for the relative changes of absorption $\Delta \xi_{260}$ at a wave length of 260 mmk.

Рис. 3. Экспериментальные графики зависимости относительного изменения величины $\overline{\Delta\tau_к}$ от содержания денатурированной ДНК в смеси

Кривая *1*: $C_{ДНК}$=10 мкг/мл; $C_{АО}$=5·10⁻⁶ М; фильтр КС-10. Кривая *2*: $C_{ДНК}$ = 5 мкг/мл; $C_{АО}$=5·10⁻⁶ М; фильтр КС-15. Кривая *3*: $C_{ДНК}$=5 мкг/мл; $C_{АО}$=5·10⁻⁶ М; фильтр ОС-13. На кривой *1* кружками отложены соответствующие относительные изменения поглощения $\overline{\Delta\xi_{260}}$ при длине волны 260 ммк

/Translator's note. The original legend is attached at the left for a comparison of figures. Probable meanings of abbreviations:

C -- content
OS -- orange light filter_/

$$\frac{\tau_K^T - \tau_K^{20'}}{\tau_K^{T_{max}} - \tau_K^{20'}} \; ; \; \frac{\varepsilon_{360}^T - \varepsilon_{360}^{20'}}{\varepsilon_{360}^{T_{max}} - \varepsilon_{360}^{20'}}$$
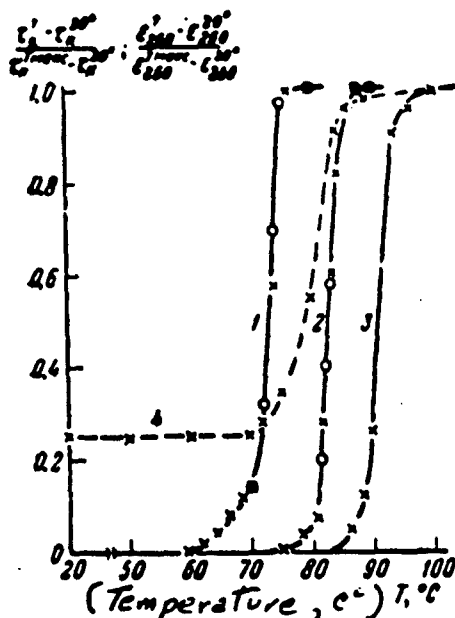
(Temperature, c°) T, °C

Рис. 4. Кривые плавления ДНК из фага Т2 при разных значениях ионной силы (μ) раствора

Растворитель — цитратный буфер. По оси абсцисс отложена температура прогрева в °C, по оси ординат—относительные изменения τ_K, измеренного через фильтр КС-10 (крестики), и относительное изменение оптической плотности ДНК (ε_{260}) в области 260 ммк (кружки).

Кривая 1: μ=0,005; $\tau_K^{20°}$=5,4·10⁻⁹ сек.; $\tau_K^{T макс}$=11,3·10⁻⁹ сек.; $\varepsilon_{260}^{20°}$=0,185; $\varepsilon_{260}^{T макс}$=0,224. Кривая 2: μ=0,08; $\tau_K^{20°}$=3,9·10⁻⁹ сек.; $\tau_K^{T макс}$=8,2·10⁻⁹ сек.; $\varepsilon_{260}^{20°}$=0,164; $\varepsilon_{260}^{T макс}$=0,210.

Кривая 3: μ=0,5; $\tau_K^{20°}$=2,7·10⁻⁹ сек.; $\tau_K^{T макс}$=4,3·10⁻⁹ сек. Кривая 4: μ=0,5; кривая была снята при медленном охлаждении (ренатурации) раствора ДНК, предварительно прогретого до 100°. Для всех кривых концентрация красителя равна 5·10⁻⁶ М. концентрация ДНК—10 мкг/мл

Figure 4. Melting curves of DNA from T2 phage at various values of ionic strength (μ) of the solution.

Solution -- citrate buffer. Along the axis of abscissas -- the temperature of heating in °C, along the axis of ordinates -- the relative change of τ_K, measured through the KS-10 filter (cross), and the relative change of optical density of DNA (ε_{260}) in the field of 260 mmk (circle).

Curve 1: μ =0.005; $\tau_K^{20°}$ = 5.4 · 10⁻⁹ sec; $\tau_K^{T max}$ = 11.3 · 10⁻⁹ sec; $\varepsilon_{360}^{20°}$ = 0.185; $\varepsilon_{260}^{T max}$ = 0.224. Curve 2: μ = 0.08; $\tau_K^{20°}$ = 3.9 · 10⁻⁹ sec; $\tau_K^{T max}$ = 8.2 · 10⁻⁹ sec; $\varepsilon_{360}^{20°}$ = 0.164; $\varepsilon_{260}^{T max}$ = 0.210.

Curve 3: μ = 0.5; $\tau_K^{20°}$ = 2.7 · 10⁻⁹ sec; $\tau_K^{T max}$ = 4.3 · 10⁻⁹ sec.

Curve 4: μ = 0.5; the curve was taken during the slow cooling (renaturing) of the solution of DNA, preliminarily heated up to 100°. For all the curves the concentration of dye equals 5 · 10⁻⁶ M, concentration of DNA -- 10 mkg/ml.